APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1997, p. 4657–4663 Vol. 63, No. 12
0099-2240/97/$04.00+0

Transient Accumulation of Glycine Betaine and Dynamics of Endogenous Osmolytes in Salt-Stressed Cultures of *Sinorhizobium meliloti*

ROLAND TALIBART,† MOHAMED JEBBAR, KAMILA GOUFFI, VIANNEY PICHEREAU, GWENOLA GOUESBET,‡ CARLOS BLANCO, THÉOPHILE BERNARD, AND JEAN-ALAIN POCARD*

Groupe Membranes et Osmorégulation, UPRES-A CNRS 6026, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes, France

Received 16 July 1997/Accepted 12 September 1997

The fate of exogenously supplied glycine betaine and the dynamics of endogenous osmolytes were investigated throughout the growth cycle of salt-stressed cultures of strains of *Sinorhizobium meliloti* which differ in their ability to use glycine betaine as a growth substrate, but not as an osmoprotectant. We present ^{13}C nuclear magnetic resonance spectral and radiotracer evidence which demonstrates that glycine betaine is only transiently accumulated as a cytoplasmic osmolyte in young cultures of wild-type strains 102F34 and RCR2011. Specifically, these strains accumulate glycine betaine as a preferred osmolyte which virtually prevents the accumulation of endogenous osmolytes during the lag and early exponential phases of growth. Then, betaine levels in stressed cells decrease abruptly during the second half of the exponential phase. At this stage, the levels of glutamate and the dipeptide *N*-acetylglutaminylglutamine amide increase sharply so that the two endogenous solutes supplant glycine betaine in the ageing culture, in which it becomes a minor osmolyte because it is progressively catabolized. Ultimately, glycine betaine disappears when stressed cells reach the stationary phase. At this stage, wild-type strains of *S. meliloti* also accumulate the disaccharide trehalose as a third major endogenous osmolyte. By contrast, glycine betaine is always the dominant osmolyte and strongly suppresses the buildup of endogenous osmolytes at all stages of the growth cycle of a mutant strain, *S. meliloti* GMI766, which does not catabolize this exogenous osmoprotectant under any growth conditions.

Glycine betaine (*N*,*N*,*N*-trimethylglycine = betaine) is a very powerful osmoprotectant in *Sinorhizobium meliloti*, the root symbiont of the legume crop alfalfa. Indeed, exogenously supplied glycine betaine strongly stimulates the growth of the free-living bacterium and enhances nitrogen fixation activity in nodulated alfalfa seedlings subjected to salt stress (1, 25, 28). Furthermore, the uptake of glycine betaine is strongly stimulated under osmotic stress, both in the free-living bacterium and in isolated bacteroids (1, 12). However, in contrast to well-studied models, such as *Escherichia coli* and *Bacillus subtilis*, which only use glycine betaine as a nonmetabolizable osmolyte, *S. meliloti* can use glycine betaine either as a growth substrate or as a cytoplasmic osmolyte, depending on the osmolarity of the growth medium (1, 2, 5, 13, 17). An example of this dual utilization was provided by a labelling experiment in which micromolar amounts of [^{14}C]betaine were fed to two cultures (stressed and unstressed) of *S. meliloti* 102F34 which were initially grown in the absence of glycine betaine. Catabolism was very active in the unstressed cells but was barely detectable in the stressed culture. Clearly, this indicates that glycine betaine preferably accumulates as a cytosolic osmolyte in stressed cells and provides further evidence of the key role of this compound in osmoregulation in *S. meliloti* (1).

In the absence of osmotic stress, glycine betaine, choline, and several choline esters, such as phosphorylcholine and acetylcholine, strongly stimulate their own catabolism (i.e., their assimilation via the glycine betaine demethylation pathway) in *S. meliloti* and *Pseudomonas aeruginosa* (21, 25, 30). Choline-containing compounds also confer high levels of stress tolerance to these species because they act as precursors to glycine betaine, which is the true osmoprotectant. Generally, exogenous levels of glycine betaine which provide maximal osmoprotection to salt-stressed cells of betaine-demethylating bacteria (about 0.5 mM) have little effect on betaine catabolism in unstressed cells because they are significantly lower than the levels required to fully activate the glycine betaine demethylation pathway in unstressed cells (10 to 20 mM) (1, 10, 29, 30). However, it has been established that glycine betaine, supplied at very low levels (about 50 μM), can rapidly reach submolar cytoplasmic concentrations in *S. meliloti* subjected to a sudden osmotic upshift (24). Thus, there is a possibility, which has not yet been explored, that high levels of cytosolic betaine in *S. meliloti* may be regulatory and could reverse the strong osmotic inhibition of betaine catabolism in stressed cells grown with minimal levels of the osmoprotectant. In other words, there is some uncertainty about the ability of betaine-demethylating bacteria, such as *S. meliloti*, to accumulate glycine betaine as a cytosolic osmolyte during long periods of osmotic stress.

In this study, we followed the dynamics of major organic osmolytes and investigated the fate of exogenous glycine betaine throughout the growth cycles of strains of *S. meliloti* which differ in their ability to use glycine betaine as a growth substrate, but not as an osmoprotectant. We present spectral

* Corresponding author. Mailing address: Groupe Membranes et Osmorégulation, UPRES-A CNRS 6026, Université de Rennes 1, Campus de Beaulieu, Av. du Général Leclerc, F 35042 Rennes, France. Phone and Fax: 33 (0)2 99 28 61 40. E-mail: pocard@univ-rennes1.fr.

† Present address: Institut Universitaire de Technologie, 22000 Saint Brieuc, France.

‡ Present address: Department of Molecular and Cellular Biology, University of Aberdeen, Aberdeen, AB25 2ZD, United Kingdom.

and radiotracer evidence demonstrating that betaine is only transiently accumulated as a cytoplasmic osmolyte in young cultures of two wild-type strains, but is durably accumulated as a dominant osmolyte throughout the growth cycle of a mutant strain, *S. meliloti* GMI766, which does not catabolize betaine (13). The shift in osmolyte preference from exogenous glycine betaine to osmolytes synthesized de novo in wild-type *S. meliloti* is discussed below in relation to the growth status of a species which must cope with osmotic stress by using a beneficial osmolyte which can activate its own catabolism (30).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. meliloti* 102F34 (9) and RCR2011 (= SU47) are standard laboratory strains. Strain GMI766 is a derivative of RCR2011 in which unspecified *gbc* (glycine betaine catabolism) genes have been deleted (13). The carbon- and nitrogen-free mineral base of the media used in this study was S medium (23). Strains were maintained on plates containing MSY (S medium plus mannitol and yeast extract [each at a concentration of 1 g/liter]). In LAS minimal medium, sodium lactate and sodium aspartate (each at a concentration of 10 mM) were substituted for mannitol and yeast extract. Betaine, lactate, and aspartate stock solutions (Sigma Chimie, St.-Quentin-Fallavier, France) were sterilized by filtration.

Bacteria were grown aerobically at 30°C on a gyratory shaker (125 rpm). Inocula were grown overnight in MSY, concentrated to an A_{420} of 10, and used at a final concentration of 1% (vol/vol). Growth was monitored spectrophotometrically at 420 nm by using a Varian 2000 spectrophotometer. Protein contents were determined as described by Lowry et al. (20) by using bovine serum albumin as the standard.

^{13}C NMR spectral determination of intracellular osmolytes. Cultures used in nuclear magnetic resonance (NMR) experiments were grown in LAS minimal medium containing 0.5 M NaCl with or without 1 mM betaine supplied as an exogenous osmoprotectant. Ethanolic extraction of cellular osmolytes and sample preparation for NMR spectral analysis were performed essentially as previously reported (34). Natural-abundance ^{13}C NMR spectra were recorded at 75.4 MHz in the Fourier transform mode by using a Bruker model AM-300WB spectrometer. Each sample was prepared from a fixed amount of cells (2,000 A_{420} units; i.e., 150 mg of protein/sample) by using 1,024 acquisitions, so that direct visual comparisons of osmolyte profiles could be made. Osmolytes were identified by comparing the chemical shifts in the spectra with those of synthetic compounds and/or previously published values (31, 34).

Fate of [^{14}C]betaine in stressed cultures of *S. meliloti*. [*methyl*-^{14}C]glycine betaine was prepared enzymatically from [*methyl*-^{14}C]choline (2.07 GBq/mmol; NEN Research Products, Les Ulis, France) as previously described (30). The accumulation and fate of [*methyl*-^{14}C]glycine betaine in osmotically stressed cultures of *S. meliloti* were investigated as follows. Strains were inoculated into LAS minimal medium containing 0.5 M NaCl and 1 mM [^{14}C]glycine betaine (1.67 MBq/mmol). The experiments were conducted at 30°C in sealed 150-ml Erlenmeyer flasks filled to a maximum of 1/10th of their capacity and shaken at 120 rpm. A 2-ml vial was hooked to each rubber seal. This vial contained a piece of Whatman no. 1 filter paper (2 by 1 cm) moistened with 0.2 ml of 5 M KOH to trap evolved $^{14}CO_2$. Growth was monitored by measuring the A_{420} of the cultures. Duplicate samples (1 ml) were harvested periodically by centrifugation (12,000 × *g*) and washed twice in 1 ml of iso-osmotic LAS minimal medium without betaine. The cell pellets were extracted three times in 80% ethanol, and the radioactivity in the ethanol-soluble fraction (ESF) and the ethanol-insoluble fraction (EIF) was determined as previously described (1, 34). Then, glycine betaine and radiolabelled metabolites in the ESF were identified by using high-voltage electrophoresis and/or paper chromatography (1, 34).

Glutamate, NAGGN, and trehalose determinations. The cytoplasmic levels of glutamate and the dipeptide *N*-acetylglutaminylglutamine amide (NAGGN) were determined by high-performance liquid chromatography analysis of the soluble fractions (34). Trehalose was measured colorimetrically by the anthrone method as previously described (17, 34). The intracellular content of organic osmolytes is reported below per milligram of cellular protein, which was determined as described by Lowry et al. (20). Cytoplasmic volumes were determined as previously described (34). All experiments were repeated at least three times with less than 10% error.

RESULTS

Variations in osmolyte composition during the growth cycle of wild-type strains of *S. meliloti*. To determine whether *S. meliloti* was able to catabolize exogenously supplied glycine betaine at a significant rate during long periods of osmotic stress, wild-type strains 102F34 and RCR2011 were grown in LAS medium containing 0.5 M NaCl and 1 mM glycine betaine, which provides maximal osmoprotection (stimulation of growth) to these strains (1). Samples were harvested at four different stages of the growth cycle of the bacteria, the early, mid-, and late exponential phases and the stationary phase of growth. Then, ^{13}C NMR spectroscopy was employed to identify all of the osmolytes that had accumulated to significant levels in the cells. Strains 102F34 and RCR2011 behaved in similar ways under our experimental conditions. As described previously, stressed cultures of *S. meliloti* grown without betaine synthesized and accumulated two major organic osmolytes, L-glutamate and the dipeptide NAGGN, during the early and mid-exponential phases of growth and also accumulated high levels of trehalose during the stationary phase (31, 34).

The spectral data obtained with strain 102F34 grown under osmotic stress in the presence of glycine betaine are presented in Fig. 1. Strikingly, only minor amounts of glutamate, but no NAGGN, were detected in betaine-grown cells harvested in the early exponential phase. In this case, exogenously supplied glycine betaine was by far the dominant cytoplasmic osmolyte (Fig. 1A). Clearly, these data indicate that (i) the transport and accumulation of readily available glycine betaine are a mechanism of osmoregulation preferred over de novo synthesis of endogenous osmolytes and (ii) cytosolic glycine betaine strongly suppresses the buildup of endogenous osmolytes (glutamate and NAGGN) in young cultures of *S. meliloti.*

In addition to glycine betaine and glutamate, cells harvested in the mid-exponential phase of growth also contained appreciable levels of NAGGN and traces of trehalose (Fig. 1B). Then, betaine levels decreased markedly in late exponential phase. Consequently, glutamate and NAGGN were the dominant solutes, and glycine betaine became a secondary osmolyte in these cells, which also contained trace levels of trehalose (Fig. 1C). Ultimately, glycine betaine disappeared from the spectrum of cells harvested in stationary phase, which also contained high levels of trehalose in addition to glutamate and NAGGN (Fig. 1D). Interestingly, this spectrum was similar to that of stationary-phase cells grown under osmotic stress without glycine betaine (31, 34).

In summary, the spectral data in Fig. 1 clearly demonstrate that the accumulation of glycine betaine is developmentally regulated in wild-type strains of *S. meliloti*; i.e., glycine betaine is preferably accumulated in young cultures and is then progressively replaced by osmolytes synthesized de novo (glutamate, NAGGN, and trehalose) during late-exponential-phase growth and in the stationary phase.

Fate of [^{14}C]glycine betaine and dynamics of endogenous osmolytes in two wild-type strains of S. meliloti. A radiotracer experiment was performed to determine whether the changes in osmolyte composition observed in wild-type strains of *S. meliloti* grown with glycine betaine (Fig. 1) were due to the substitution of endogenously synthesized osmolytes for the transported osmoprotectant or to the possible metabolism of glycine betaine in older cultures. *S. meliloti* 102F34 and RCR2011 were inoculated into LAS minimal medium supplemented with 0.5 M NaCl plus 1 mM [*methyl*-^{14}C]glycine betaine. Samples were removed periodically, and the distribution of the radioactivity in the growth medium, the cellular ESF and EIF, and $^{14}CO_2$ was determined as described above. Again, similar results were obtained with the two strains.

Figure 2 shows that about 15% of the supplied radioactivity was taken up from the growth medium during the lag phase, i.e., before the stressed culture of *S. meliloti* 102F34 started to grow. Then, the radioactivity in the medium decreased linearly with time and increasing cell density throughout the exponential phase. Consequently, less than 8% of the supplied radio-

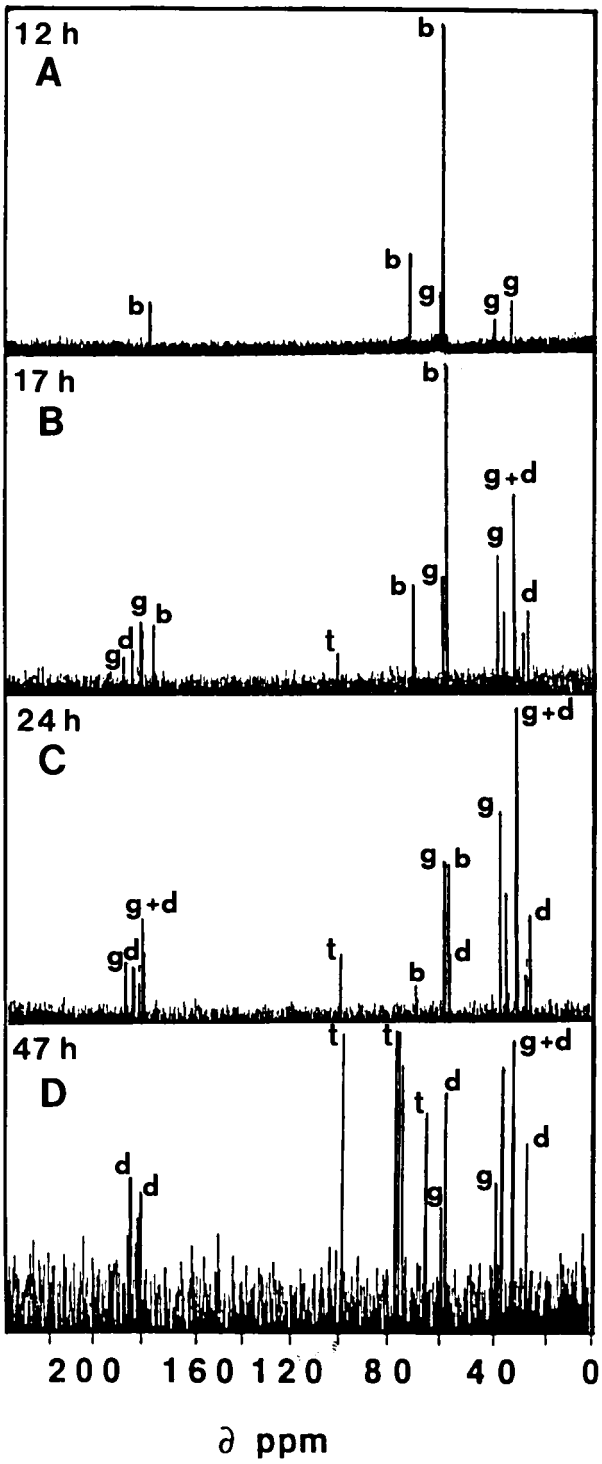

FIG. 1. Effect of exogenous glycine betaine on the osmolyte composition of *S. meliloti* wild-type strain 102F34. The cells were grown in LAS minimal medium containing 0.5 M NaCl and 1 mM glycine betaine. Samples were harvested 12, 17, 24, and 47 h after inoculation, i.e., in the early exponential phase (A), in the mid-exponential phase (B), during transition to the stationary phase (C), and in the stationary phase (D), respectively. Natural-abundance ^{13}C NMR analysis of ethanolic extracts was performed as described in Materials and Methods. All spectra (1,024 scans) were obtained from a fixed amount of cells (2,000 A_{420} units) and are shown at the same scale. Resonances (peaks) from glycine betaine (b), L-glutamate (g), the dipeptide NAGGN (d), and trehalose (t) are indicated when these osmolytes were detected in the extracts. Similar spectra were obtained when *S. meliloti* wild-type strain RCR2011 was grown and assayed under similar conditions.

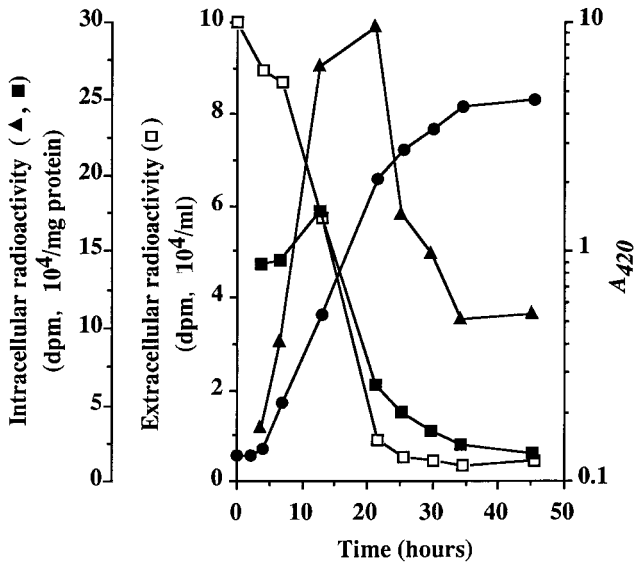

FIG. 2. Fate of [^{14}C]glycine betaine during the growth cycle of a stressed culture of *S. meliloti* wild-type strain 102F34. The culture was grown in LAS minimal medium containing 0.5 M NaCl and 1 mM [*methyl*-^{14}C]glycine betaine (1.67 MBq/mmol), supplied as an exogenous osmoprotectant. Cells were harvested and extracted as described in the text. Symbols: ●, growth expressed as A_{420}; □, radioactivity in the growth medium; ■, radioactivity in the ESF; ▲, radioactivity in the EIF.

activity was recovered in the growth medium 25 h after inoculation, i.e., at the end of the exponential phase.

Interestingly, the supplied radiocarbon was quantitatively recovered as [^{14}C]glycine betaine in the ESF (the cytoplasmic fraction) during the earliest stages of the culture. Indeed, no significant amounts or small amounts of radioactivity were recovered in the EIF before the culture reached an optical density (A_{420}) of 0.2 U, i.e., up to about 6 h after inoculation. In other words, exogenous glycine betaine was accumulated as a stable cytoplasmic osmolyte during the lag phase and the first few hours of exponential growth. Then, its metabolism was initiated (Fig. 2). However, the radioactivity in the ESF continued to increase parallel to the cell density increase during the first half of the exponential phase, i.e., up to about 13 h postinoculation. Then, it decreased very abruptly parallel to decreasing radioactivity in the growth medium. Meanwhile, the radioactivity in the EIF reached its maximal level during transition to stationary phase and decreased steadily in the ageing culture because the radiocarbon was progressively transferred to $^{14}CO_2$. Ultimately, 46 h after inoculation, 50% of the supplied radioactivity was recovered in $^{14}CO_2$ (data not shown).

In summary, the data from this radiotracer experiment confirm that glycine betaine acts as a cytoplasmic osmolyte in *S. meliloti*. They also indicate that the metabolism of the accumulated betaine started early in the exponential phase and continued in the growing and ageing culture, until the supply of the exogenous osmoprotectant was exhausted. In other words, glycine betaine was only transiently accumulated in stressed cells of wild-type strains of *S. meliloti*.

To evaluate their contributions to osmotic adjustment, glycine betaine and endogenously synthesized osmolytes were also quantified at all stages of the growth cycle of *S. meliloti* 102F34 grown in the presence of 0.5 M NaCl plus 1 mM [*methyl*-^{14}C]glycine betaine. The radiolabelled betaine was readily quantified following electrophoretic analysis of the ESF. Figure 3 shows that the level of cytosolic betaine in

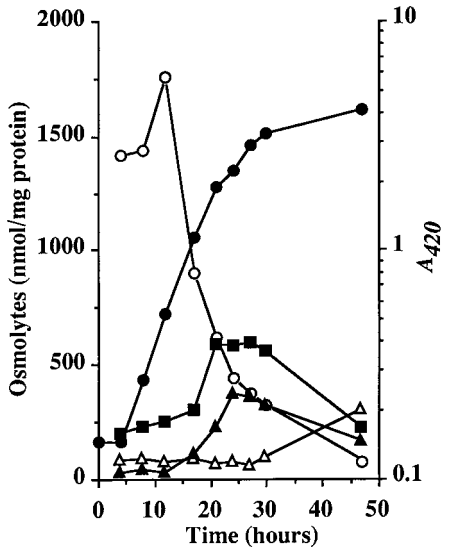

FIG. 3. Variations in the osmolyte composition of *S. meliloti* wild-type strain 102F34 during growth at high osmolarity in the presence of glycine betaine. A culture was grown in LAS minimal medium containing 0.5 M NaCl and 1 mM [*methyl*-^{14}C]glycine betaine (1.67 MBq/mmol). Cells were extracted in ethanol, and cytoplasmic osmolytes were quantified as described in Materials and Methods. Symbols: ●, growth expressed as A_{420}; ○, glycine betaine; ■, glutamate; ▲, NAGGN; △, trehalose.

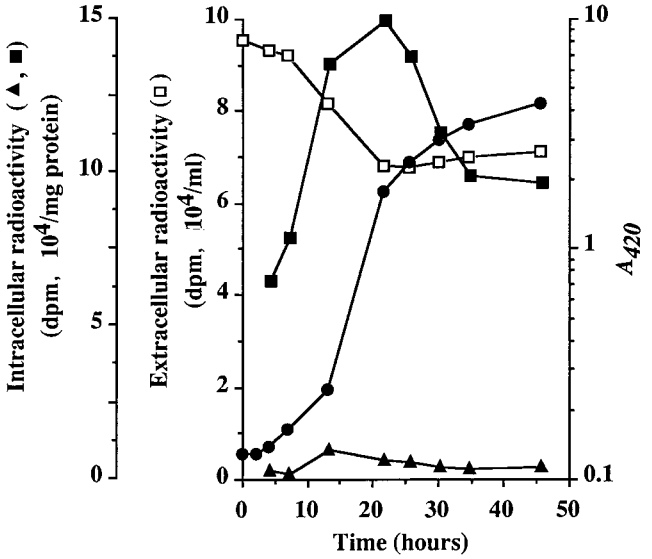

FIG. 4. Accumulation of exogenous glycine betaine during the growth cycle of mutant *S. meliloti* GMI766, which does not catabolize this osmoprotectant. Cells were grown in LAS minimal medium containing 0.5 M NaCl and 1 mM [*methyl*-^{14}C]glycine betaine (1.67 MBq/mmol), harvested, and processed as described in the text. Symbols: ●, growth expressed as A_{420}; □, radioactivity in the growth medium; ■, radioactivity in the ESF; ▲, radioactivity in the EIF.

stressed cells of *S. meliloti* increased from 1,400 nmol/mg of protein in the lag and early log phases to 1,750 nmol/mg of protein at mid-log phase and then decreased very abruptly. The maximal level of glycine betaine in mid-log-phase cells corresponded to a calculated cytosolic concentration of about 0.5 M. Ultimately, the betaine levels in stationary-phase cells represented less than 10% of this maximal level. Strikingly, a dramatic increase in glutamate and NAGGN levels was initiated when the cytosolic betaine level started to decrease. Indeed, during the second half of the exponential phase, the glutamate and NAGGN levels increased 2- to 3-fold and 8- to 10-fold, respectively. Consequently, the two endogenous osmolytes became the dominant solutes during transition to stationary phase and compensated osmotically for the sharp decrease in the intracellular betaine level observed in the ageing culture. Lastly, the levels of trehalose, which were stable throughout the exponential phase, increased during transition to stationary phase, confirming previous observations that trehalose is a major osmolyte in ageing and nongrowing bacterial cultures (5, 17, 32, 34).

Glycine betaine is durably accumulated as a cytoplasmic osmolyte in stressed cultures of *S. meliloti* GMI766. The fate of the osmoregulated pool of glycine betaine was also investigated in *S. meliloti* GMI766, a deletion derivative of RCR2011 which is unable to catabolize glycine betaine (13). Strain GMI766 was inoculated into LAS medium supplemented with 0.5 M NaCl plus 1 mM [*methyl*-^{14}C]glycine betaine, which also conferred maximal osmoprotection to this strain (data not shown). Figure 4 shows that the radioactivity in the growth medium decreased in proportion to increasing cell number (A_{420}) during the exponential phase, but did not decrease thereafter. Indeed, 80% of the supplied betaine remained in the growth medium when GMI766 had reached the stationary phase (Fig. 4), while only trace levels of radioactivity were found in the medium of older cultures of the wild-type strains (Fig. 2). The radioactivity in the ESF increased in proportion to increasing cell density during the exponential phase. Then, it decreased by one-third during transition to stationary phase and remained stable in the stationary phase. Furthermore, as expected from a strain which does not metabolize glycine betaine (13), no significant amount of radioactivity was ever detected in the EIF (Fig. 4) or in $^{14}CO_2$, and only radiolabelled betaine itself was identified in the growth medium and in the ESF (data not shown). Thus, the cytosolic betaine in GMI766 was easily assessed by measuring the radioactivity of the ESF. The curve expressing its levels (in nanomoles of betaine per milligram of protein) could be rigorously superimposed on the curve representing the ESF in Fig. 4. In other words, betaine was durably accumulated in stressed cells of *S. meliloti* GMI766. Interestingly, the maximal cytosolic levels were reached during the exponential phase, i.e., in growing cells. These levels were similar to those observed in mid-exponential phase in strains 102F34 and RCR2011, i.e., before the catabolism of the osmolyte started in these strains (about 1,700 nmol of betaine/mg of protein) (Fig. 3). Then, the betaine levels in GMI766 decreased about one-third during transition to stationary phase, most probably because lower turgor is required in nongrowing cells than in growing cells (18).

Lastly, the ^{13}C NMR spectra from stressed cells of GMI766 harvested in early, mid-, and late log phases and in stationary phase were very similar (data not shown). They were also similar to the spectrum from a young culture of wild-type strain RCR2011 which was harvested in early log phase, i.e., before the metabolism of the osmoregulated pool of glycine betaine started in this strain (Fig. 1A). In other words, glycine betaine and glutamate were the only organic solutes to contribute significantly to osmostasis in stressed *S. meliloti* GMI766 growing in the presence of the nonmetabolizable betaine. Glutamate levels were eight to nine times lower than betaine levels (data not shown).

DISCUSSION

Glycine betaine is often viewed as an osmolyte which is durably accumulated in stressed cells and is released into the environment following a sudden osmotic downshift. This perception usually stems from the fact that well-studied plants and bacteria, such as halophytes, enteric bacteria, and many other bacteria, do not catabolize glycine betaine under any growth conditions (2, 5, 26). Unlike these organisms, *S. meliloti* uses glycine betaine either as a source of carbon and nitrogen or as a cytoplasmic osmolyte, depending on the osmolarity of its growth medium (1). However, because glycine betaine stimulates its own catabolism in unstressed cells (30), there has been some uncertainty about the ability of *S. meliloti* to accumulate glycine betaine as a cytoplasmic osmolyte during long periods of osmotic stress. Therefore, in this study, we followed the fate of this compound and monitored the changes in osmolyte composition throughout the growth cycle of strains *S. meliloti* which differ in the ability to use glycine betaine as a growth substrate, but not as an osmoprotectant.

Our data confirm that exogenous glycine betaine is accumulated as a cytoplasmic osmolyte in *S. meliloti*. However, we show that glycine betaine is only transiently accumulated in young cultures of wild-type strains RCR2011 and 102F34, but is durably accumulated throughout the growth cycle of mutant *S. meliloti* GMI766, which does not catabolize glycine betaine (13). Furthermore, we show that high cytoplasmic levels of glycine betaine in osmotically stressed cultures of *S. meliloti* strongly suppress the buildup of endogenous osmolytes (glutamate, NAGGN, and trehalose) in cells which do not catabolize the supplied osmoprotectant. Such shifts in osmolyte preference from endogenous osmolytes to exogenous osmoprotectants, such as glycine betaine, other betaines, and proline, are well documented in bacteria and provide further evidence that these zwitterionic compounds are highly compatible solutes (5, 10, 17). Also, it is noteworthy that the sum of all organic osmolytes is lower in ageing than in young cultures of *S. meliloti* 102F34, particularly in the stationary phase (Fig. 3). This probably reflects the fact that the level of potassium (a counterion to glutamate) is consistently lower in stressed bacteria accumulating high levels of betaine (5, 33, 36), but most likely increases in proportion to the level of glutamate, which progressively supplants the catabolized betaine in 102F34. Moreover, it is acknowledged that the turgor required to drive cell growth (elongation) is greater than that required to maintain osmotic equilibrium in nongrowing cells (5, 18).

Several nonexclusive interpretations may account for the sequential accumulation of exogenous glycine betaine and endogenously synthesized osmolytes in wild-type strains of *S. meliloti* grown under osmotic stress in the presence of glycine betaine. First, the glycine betaine accumulated in stressed cells could be progressively catabolized because, as a compatible solute, it may protect enzymes involved in its own catabolism against the deleterious effects of osmotic and/or salt inhibition (5, 35, 37).

Second, it is noteworthy that high-affinity betaine uptake (K_m = 5 to 10 μM) is constitutive in *S. meliloti*, whereas it is induced at high osmolarities in many other bacteria (1, 5, 15). Furthermore, this uptake is rapidly and strongly stimulated following a sudden osmotic upshock (there is a four- to sixfold increase in uptake rate in less than 15 s). Consequently, upshocked cells of *S. meliloti* can accumulate half-molar concentrations of betaine from micromolar levels (<50 μM) of exogenous substrate in a few minutes (24). Then, growth can resume at a suboptimal rate when positive cytoplasmic turgor has been established to balance the osmotic differential between the cell and its environment (1, 5, 24). Later, during exponential growth, the metabolism of the cytoplasmic pool of glycine betaine could start because osmoadapted cells of *S. meliloti* may sense that their environment is less stressful than the environment of nonadapted cells. In contrast, stressed cells grown without betaine must synthesize endogenous osmolytes prior to resuming growth at high osmolarities. Apparently, the accumulation of glutamate occurs because of rapid modifications of the activity of enzymes involved in glutamate metabolism (3). However, the synthesis of NAGGN and trehalose requires the induction or the activation of specific genes. Obviously, the setup of these metabolic pathways requires more energy and is physiologically less efficient than the immediate activation of a constitutive betaine uptake activity following a sudden osmotic upshock (5, 24, 27, 31, 34).

Third, because it activates its own catabolism in unstressed cells and accumulates to high levels in stressed cells (1, 30), glycine betaine may also become regulatory in stressed cells of *S. meliloti*. Therefore, the fact that there is dual utilization of glycine betaine in osmoregulation and in nutrition in osmotically stressed cells of wild-type *S. meliloti* is not necessarily a metabolic contradiction. Indeed, it is well established that glycine betaine is an excellent growth substrate and a proven intermediate in the assimilation of carnitine, acetylcholine, and/or phosphorylcholine in *S. meliloti*, *Pseudomonas fluorescens*, and *P. aeruginosa*, i.e., in at least three species of the γ subclass of the *Proteobacteria* (11, 16, 21, 35). Furthermore, *P. aeruginosa* uses glycine betaine both as a sole source of carbon and nitrogen and as a cytosolic osmolyte during growth at high osmolarities (19, 29). Moreover, to avoid possible interference with constituents of rich media, the osmoprotective activity of betaines is typically evaluated in defined minimal media (in this study, a lactate-aspartate medium) which are not always optimal for bacterial growth (1, 6, 8, 15). Thus, for all of these reasons, it is not surprising that wild-type strains of *S. meliloti* use glycine betaine as an additional growth substrate once they can synthesize endogenous osmolytes.

The mechanism by which osmotic stress inhibits the catabolism of glycine betaine in young cultures of wild-type strains of *S. meliloti* remains unclear. However, environmental factors other than betaine itself and high osmolarity might direct the utilization of this compound in nutrition or in osmoregulation. As suggested above, the availability of readily metabolizable growth substrates probably plays an important role. Indeed, the nutritional status of a bacterium can affect its osmolyte composition. For example, stressed bacteria growing under nitrogen-limited conditions are biased toward accumulating osmolytes with few or no nitrogen atoms (4, 27). The composition of the growth medium can also affect the level of salinity tolerance of a bacterium. For example, *Halomonas* sp. strain SPC1 grows on glycine betaine and ammonia at salinities up to 2.0 M NaCl. In this case, glycine betaine is used as a sole source of carbon, as a cytosolic osmolyte, and as a precursor to ectoine, the prominent endogenous osmolyte in this strain. Furthermore, *Halomonas* sp. strain SPC1 can grow in the presence of up to 3.5 M NaCl when glucose is supplied as the carbon source and glycine betaine is supplied as an osmoprotectant. In this case, glycine betaine, which is accumulated to high levels, strongly reduces the accumulation of ectoine and is apparently stable, as judged from in vitro enzyme assays (6, 7). Interestingly, the accumulation of glycine betaine and ectoine is also developmentally regulated when *Halomonas* sp. strain SPC1 is grown at lower salinities (2.0 M NaCl); glycine betaine predominates over ectoine in the early exponential phase, while ectoine is more abundant in the stationary phase (6).

Like *S. meliloti*, two unidentified halophilic bacteria, strains ML-D and ML-G, also use glycine betaine as an osmoprotectant and a growth substrate at high osmolarities. Interestingly, the catabolism of glycine betaine predominates over accumulation of this compound in stressed cells; ML-D only accumulates glycine betaine as a secondary osmolyte when it is grown in the presence of more than 2.0 M NaCl, and ML-G does not accumulate glycine betaine, even when it is grown in the presence of 3.0 M NaCl, i.e., near its maximal level of salinity tolerance (4, 8). The situation with glycine betaine in ML-G resembles the situation with ectoine in *S. meliloti* 102F34 and RCR2011, in which ectoine never accumulates as a cytoplasmic osmolyte, although it is highly osmoprotective in these strains (34). This mode of osmoprotection is unusual and very intriguing because it is not directly explicable by the classical model which specifies that osmoprotectants are beneficial because they restore turgor in stressed cells and are compatible with cellular functions at high cytoplasmic concentrations (5, 8, 34, 35, 38). This general mechanistic model can explain the osmoprotective activity of glycine betaine in *S. meliloti* GMI766 and in young cultures of wild-type strains 102F34 and RCR2011. Furthermore, our data obtained with the catabolic mutant (GMI766) demonstrate that osmoprotection by glycine betaine in wild-type strains of *S. meliloti* is not linked to catabolism of this compound, as might be the case for ectoine in these strains (34).

Lastly, this research raises the question of the relevance of foiling the catabolism of glycine betaine and other betaines found in alfalfa (37) to improve their osmoprotective activity in *S. meliloti*. Strain GMI766 does not catabolize glycine betaine because as-yet-unidentified genes have been deleted in this strain (13). Consequently, glycine betaine is durably accumulated as a preferred osmolyte in the stressed mutant (Fig. 4). However, no further improvement in growth due to glycine betaine is observed at high osmolarity in GMI766 compared with the increase in growth rate and cell yield observed in stressed cultures of RCR2011 or 102F34, which ultimately catabolize the accumulated osmoprotectant. Such an improvement might be achieved under experimental conditions which remain to be determined or by using strains harboring appropriate mutations. However, we believe that preventing the catabolism of glycine betaine might not be the most judicious approach because betaines, such as stachydrine (*N*,*N*-dimethylproline), trigonelline, and possibly glycine betaine itself, also function as valuable nutritional mediators and *nod* gene inducers during the symbiosis between alfalfa and *S. meliloti* (12–14). Alternatively, the osmotolerance of *Rhizobium* species could be increased through genetic engineering of metabolic pathways encoding the synthesis of new osmolytes which do not play a crucial role in nutrition and/or in symbiosis in these species (22).

ACKNOWLEDGMENTS

This research was supported by grants from the Centre National de la Recherche Scientifique, the Ministère de l'Education Nationale et de la Recherche, and the University of Rennes 1.

We thank Jack Hamelin for performing the NMR analysis and Charles Rosenberg for providing strain GMI766.

REFERENCES

1. **Bernard, T., J.-A. Pocard, B. Perroud, and D. Le Rudulier.** 1986. Variations in the response of salt-stressed *Rhizobium* strains to betaines. Arch. Microbiol. **143:**359–364.
2. **Boch, J., B. Kempf, and E. Bremer.** 1994. Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline. J. Bacteriol. **176:**5364–5371.
3. **Botsford, J. L., and T. A. Lewis.** 1990. Osmoregulation in *Rhizobium meliloti*: production of glutamic acid in response to osmotic stress. Appl. Environ. Microbiol. **56:**488–494.
4. **Ciulla, R. A., M. R. Diaz, B. F. Taylor, and M. F. Roberts.** 1997. Organic osmolytes in aerobic bacteria from Mono Lake, an alkaline, moderately hypersaline environment. Appl. Environ. Microbiol. **63:**220–226.
5. **Csonka, L. N., and A. D. Hanson.** 1991. Prokaryotic osmoregulation: genetics and physiology. Annu. Rev. Microbiol. **45:**569–606.
6. **Cummings, S. P., and D. J. Gilmour.** 1995. The effect of NaCl on the growth of a *Halomonas* species: accumulation and utilization of compatible solutes. Microbiology **141:**1413–1418.
7. **Cummings, S. P., M. P. Williamson, and D. J. Gilmour.** 1993. Turgor regulation in a novel *Halomonas* species. Arch. Microbiol. **160:**319–323.
8. **Diaz, M. R., and B. F. Taylor.** 1996. Metabolism of methylated osmolytes by aerobic bacteria from Mono Lake, a moderately hypersaline, alkaline environment. FEMS Microbiol. Ecol. **19:**239–247.
9. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
10. **D'Souza-Ault, M. R., L. T. Smith, and G. M. Smith.** 1993. Roles of *N*-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress. Appl. Environ. Microbiol. **59:** 473–478.
11. **Fitch, W. M.** 1963. Studies on a cholinesterase of *Peudomonas fluorescens*. I. Enzyme induction and the metabolism of acetylcholine. Biochemistry **2:** 1217–1221.
12. **Fougère, F., and D. Le Rudulier.** 1990. Glycine betaine biosynthesis and catabolism in bacteroids of *Rhizobium meliloti*: effect of salt stress. J. Gen. Microbiol. **136:**2503–2510.
13. **Goldmann, A., C. Boivin, V. Fleury, B. Message, L. Lecoeur, M. Maille, and D. Tepfer.** 1991. Betaine use by rhizosphere bacteria: genes essential for trigonelline, stachydrine, and carnitine catabolism in *Rhizobium meliloti* are located on pSym in the symbiotic region. Mol. Plant Microbe Interact. **4:**571–578.
14. **Goldmann, A., L. Lecoeur, B. Message, M. Delarue, E. Schoonejans, and D. Tepfer.** 1994. Symbiotic plasmid genes essential to the catabolism of proline betaine, or stachydrine, are also required for efficient nodulation by *Rhizobium meliloti*. FEMS Microbiol. Lett. **115:**305–311.
15. **Kappes, R. M., B. Kempf, and E. Bremer.** 1996. Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of OpuD. J. Bacteriol. **178:**5071–5079.
16. **Kleber, H.-P.** 1997. Bacterial carnitine metabolism. FEMS Microbiol. Lett. **147:**1–9.
17. **Koch, A. L.** 1988. Biophysics of bacterial walls viewed as stress-bearing fabric. Microbiol. Rev. **52:**337–353.
18. **Larsen, P. I., L. K. Sydnes, B. Landfald, and A. R. Strøm.** 1987. Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. Arch. Microbiol. **147:**1–7.
19. **Lisa, T. A., C. H. Casale, and C. E. Domenech.** 1994. Cholinesterase, acid phosphatase, and phospholipase C of *Pseudomonas aeruginosa* under hyperosmotic conditions in a high-phosphate medium. Curr. Microbiol. **28:**71–76.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
21. **Lucchini, A. E., T. A. Lisa, and C. E. Domenech.** 1990. Choline derivatives increase two different acid phosphatases in *Rhizobium meliloti* and *Pseudomonas aeruginosa*. Arch. Microbiol. **153:**596–599.
22. **Neumivakin, L. V., V. P. Solovjev, A. Sokhansanj, V. A. Tilba, N. A. Moseiko, R. V. Shachbasian, and E. S. Piruzian.** 1995. Increase in osmotolerance of *Rhizobium fredii* soybean isolate BD32 by the *proB proA* operon of *Escherichia coli*. Biochem. Biophys. Res. Commun. **217:**796–801.
23. **O'Gara, F., and K. T. Shanmugam.** 1976. Regulation of nitrogen fixation by rhizobia: export of fixed N_2 as NH_4^+. Biochim. Biophys. Acta **437:**313–321.
24. **Pocard, J.-A.** 1987. La glycine bétaïne, effet osmoprotecteur, transport et métabolisme chez *Rhizobium meliloti* cultivé *in vitro* et en symbiose avec *Medicago sativa* L. Ph. D. thesis. University of Rennes 1, Rennes, France.
25. **Pocard, J.-A., T. Bernard, G. Goas, and D. Le Rudulier.** 1984. Restauration partielle, par la glycine bétaïne et la proline bétaïne, de l'activité fixatrice d'azote de jeunes plantes de *Medicago sativa* L. soumises à un stress hydrique. C. R. Acad. Sci. **298:**477–480.
26. **Rhodes, D., and A. D. Hanson.** 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **44:**357–384.
27. **Robertson, D. E., M.-C. Lai, R. P. Gunsalus, and M. F. Roberts.** 1992. Composition, variation, and dynamics of major osmotic solutes in *Methanohalophilus* strain FDF1. Appl. Environ. Microbiol. **58:**2438–2443.
28. **Sauvage, D., J. Hamelin, and F. Larher.** 1983. Glycine betaine and other structurally related compounds improve the salt tolerance of *Rhizobium meliloti*. Plant Sci. Lett. **31:**291–302.
29. **Shortridge, V. D., A. Lazdunski, and M. L. Vasil.** 1992. Osmoprotectants and phosphate regulate expression of phospholipase-C in *Pseudomonas aeruginosa*. Mol. Microbiol. **6:**863–871.
30. **Smith, L. T., J.-A. Pocard, T. Bernard, and D. Le Rudulier.** 1988. Osmotic

control of betaine biosynthesis and degradation in *Rhizobium meliloti*. J. Bacteriol. **170:**3142–3149.
31. **Smith, L. T., and G. M. Smith.** 1989. An osmoregulated dipeptide in stressed *Rhizobium meliloti*. J. Bacteriol. **171:**4714–4717.
32. **Smith, L. T., G. M. Smith, M. R. D'Souza, J.-A. Pocard, D. Le Rudulier, and M. A. Madkour.** 1994. Osmoregulation in *Rhizobium meliloti*: mechanism and control by other environmental signals. J. Exp. Zool. **268:**162–165.
33. **Sutherland, L., J. Cairney, M. J. Elmore, I. R. Booth, and C. F. Higgins.** 1986. Osmotic regulation of transcription: induction of the *proU* betaine transport gene is dependent on accumulation of intracellular potassium. J. Bacteriol. **168:**805–814.
34. **Talibart, R., M. Jebbar, G. Gouesbet, S. Himdi-Kabbab, H. Wroblewski, C. Blanco, and T. Bernard.** 1994. Osmoadaptation in rhizobia: ectoine-induced salt tolerance. J. Bacteriol. **176:**5210–5217.
35. **Warr, S. R. C., R. H. Reed, and W. D. P. Stewart.** 1988. The compatibility of osmotica in cyanobacteria. Plant Cell Environ. **11:**137–142.
36. **Whatmore, A. M., J. A. Chudek, and R. H. Reed.** 1990. The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. J. Gen. Microbiol. **136:**2527–2535.
37. **Wood, K. V., K. J. Stringham, D. L. Smith, J. J. Volenec, K. L. Hendershot, K. A. Jackson, P. J. Rich, W. J. Yang, and D. Rhodes.** 1991. Betaines of alfalfa. Characterization by fast atom bombardment and desorption chemical ionization mass spectrometry. Plant Physiol. **96:**892–897.
38. **Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero.** 1982. Living with water stress: evolution of osmolyte systems. Science **217:** 1214–1222.